Wnt signaling: why is everything so negative?

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The Wnt proteins constitute a family of secreted glycoproteins the members of which have essential signaling roles during embryogenesis. The recent identification of several new regulators of this signal transduction pathway have revealed unexpectedly intricate levels of constraint on Wnt-dependent gene activation, and studies in developing embryos and in cell culture systems have allowed a more complete understanding of the functional and biochemical interactions between components of this evolutionarily conserved pathway.

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Abbreviations

APC	adenomatous polyposis coli protein
β-cat	β-catenin
BMP	bone morphogenic protein
CRD	cystein rich domain
CKII	casein kinase II
Dsh	Dishevelled
GSK-3β	glycogen synthase kinase-3β
LEF/TCF	lymphoid enhancer factor/T-cell factor
MBT	mid-blastula transition
PI	phosphatidylinositol
PKC	protein kinase C

Introduction

Signal transduction pathways are elegant systems by which cells and organisms can amplify subtle signals to generate robust responses, but it is essential that signalling pathways are not activated spuriously. To that apparent end the molecule at the heart of the Wnt signaling pathway, β-catenin (or Armadillo, the *Drosophila* homolog), is maintained under constant negative regulation by the constitutively active glycogen synthase kinase-3β (GSK-3β). Only by repressing this perpetual inhibition can a Wnt signal be transduced (reviewed in [1]). In addition, several secreted factors inhibit Wnt signaling [2,3•] (reviewed in [4]), as do other signaling pathways [5,6]. Data accumulated during the past 18 months have filled many gaps in our understanding of signal transduction and have revealed further levels of negative regulation of Wnt signaling. In this review we will concentrate our attention on newly identified components of the pathway as well as on the positive and negative regulation of Wnt signal transduction.

Wnt signaling basics

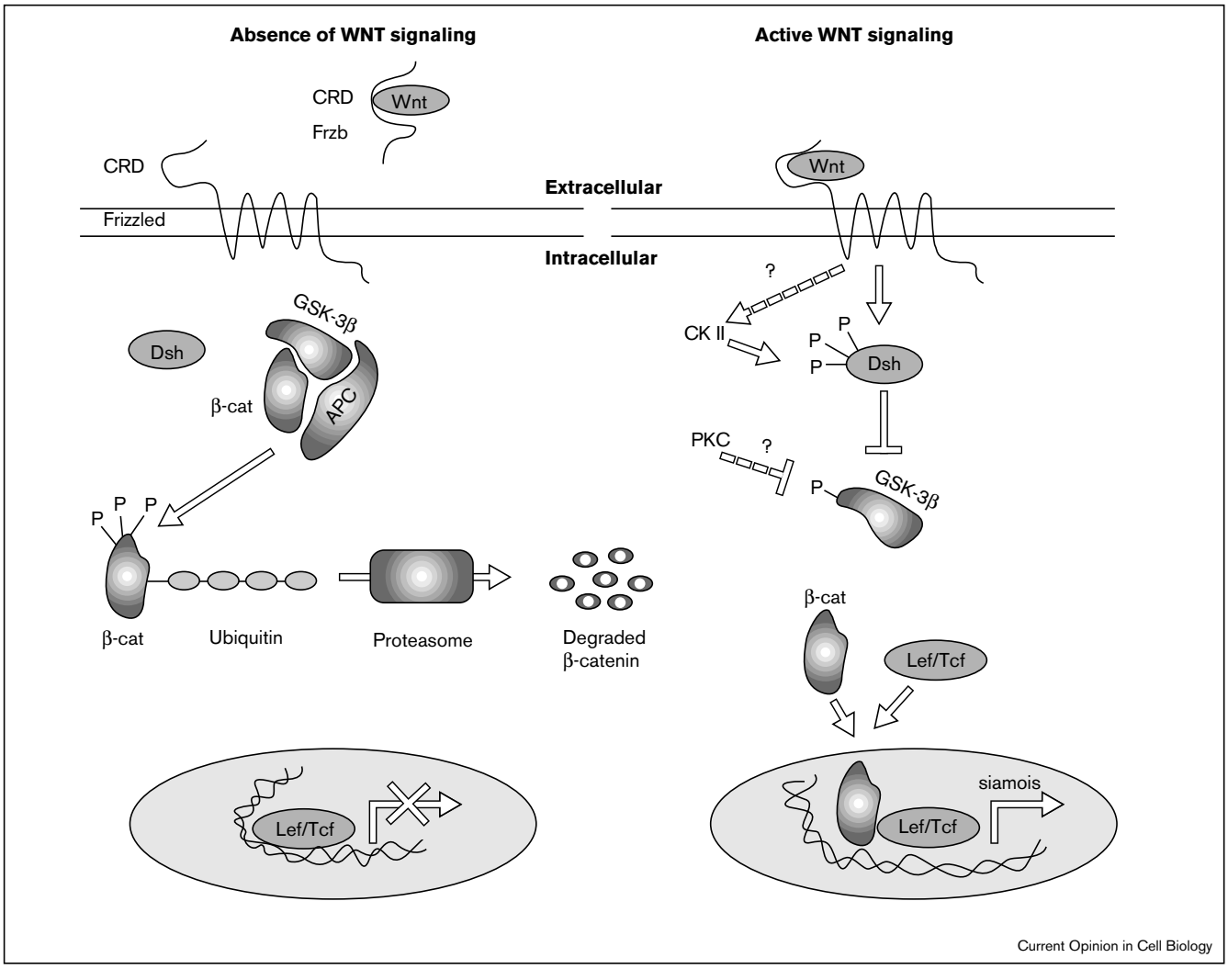
In the absence of Wnt signaling, GSK-3β causes the free cytosolic pool of β-catenin to be degraded (Figure 1; [7–9], reviewed in [1]). Association of appropriate members of the Wnt (reviewed in [10]) and Frizzled [11] protein families leads to activation of the signaling pathway (Figure 1; [12,13,14•]). The Dishevelled (Dsh) protein, the most proximal intracellular component known in the pathway [15] is somehow activated, phosphorylated in its central domain by casein kinase II (CKII) [16•], and is recruited to cell membranes [13,17]. In spite of changes in localization and phosphorylation state, it is not clear how Dishevelled is activated. Overexpression of either Dishevelled or the *Drosophila* Frizzled-2 protein is sufficient to lead to phosphorylation by CKII, but does not necessarily lead to activation of the protein [16•]. Activation of Dishevelled somehow leads to the inhibition of GSK-3β, possibly involving protein kinase C (PKC) phosphorylation of an amino-terminal serine residue of GSK-3β [18]. As newly translated β-catenin accumulates because of reduced GSK-3β activity, β-catenin can then interact with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of architectural transcription factors [19,20,21••] in the nucleus [22•]. In the nucleus the complex binds consensus LEF/TCF sites in promoters and induces transcription of Wnt-responsive genes (Figure 1; [20,23,24•], reviewed in [25]). Several recent reviews have described the 'classical' Wnt-1 signal transduction pathway and the roles of Wnt signaling during development in greater detail [4,10,26,27].

Additional attention has recently been focused upon alternative Wnt and Frizzled signaling pathways. Examples of Wnt/Frizzled signaling pathways that diverge from the model presented in Figure 1 have been observed in *Xenopus* [2] (reviewed in [28]), zebrafish [29•,30••], *Drosophila* [31] (reviewed in [32]), and *Caenorhabditis elegans* [33,34••]. It is not clear whether the divergent pathways in these organisms represent a second cohesive system by which a subset of Wnt signals are transduced, or whether animals with different developmental strategies have tailored components of the well-conserved 'Wnt-1 pathway' (Figure 1) to their own needs.

Antagonists abound

Models invoking morphogenic gradients of secreted signaling factors to explain patterning of developing tissues have been a favorite among developmental biologists for a century; however, the recent identification of several secreted antagonists of bone morphogenic protein (BMP), reviewed in [35]) and Wnt (below) signaling add a new dimension to our understanding of these patterning

Figure 1



The 'classical' Wnt-1 signaling pathway. In the absence of Wnt-1 class signals, or if Wnts are bound and inhibited by members of the Frzb family, a complex of GSK-3β and APC target β-catenin for degradation. Low levels of cytosolic β-cat are insufficient to be translocated to the nucleus and associate with LEF/TCF architectural transcription factors. LEF/TCF are likely bound to promoters in the absence of β-cat and repress basal transcription. Wnt likely binds to the extracellular cysteine-rich domain of Frizzled and leads to the hyperphosphorylation of Dsh by CKII. CKII phosphorylation is not sufficient to activate Dsh; other events are also required. Activated Dsh inhibits GSK-3β function by a poorly understood mechanism that may involve PKC phosphorylation of GSK-3β. β-cat levels rise as the result of decreased rates of degradation mediated by GSK-3β. β-cat appears to be translocated into the nucleus independent of association with LEF/TCF. Inside the nucleus, β-cat may alter the ability of LEF/TCF to bend DNA and may relieve LEF/TCF-dependent transcriptional repression. A carboxy-terminal domain of β-cat then activates transcription.

events. It seems likely that secreted antagonists of other signaling proteins will be revealed in the future.

The Frzb family

The Frzb proteins are expressed in a wide range of tissues in embryos and adults and seem to act by binding to secreted Wnt proteins and preventing productive interactions between Wnt proteins and Frizzled proteins. (reviewed in [4]). The prototypic member of this family, FRZB, was cloned by researchers interested in a chondrogenic activity observed in a purified protein fraction from bovine cartilage [36]. Microsequence analysis of the purified protein followed by degenerate PCR (polymerase chain reaction) cloning and library screening resulted in the isolation of a full-length cDNA encoding a cysteine-rich domain (CRD) with significant similarity to the extracellular amino-terminal domain of Frizzled receptors [36]—a region now known to be important for binding of Wnt [12,37•]. Subsequently, other groups cloned members of the Frzb family by differential screens [38••,39,40], isolation of proteins co-purifying with mitogenic fractions from fibroblastic cell lines [41•,42], activity screens [43] and by homology to known Frizzled receptors [44•]. At least five distinct members of the Frzb family have been cloned to date, with homologs isolated from several vertebrate species.

Members of the Frzb family seem to be surprisingly similar in their specificity for inhibiting Wnt signaling. The three members tested to date, Frzb-1/sFRP-3, sFRP-2, FRP/sFRP-1, have been shown to interact with Wnt-1 [37•,38••,45••], Wnt-8 [45••] or Wingless [44•] by indirect immunofluorescence or coimmunoprecipitation. In various assays for Wnt function in *Xenopus*, Frzb-1/fiz [38••,42,45••] and FRP/sFRP-1 [41,44•] block the activities of Wnt-1, Wnt-8 and Wingless, all members of the 'Wnt-1 functional class' [46,47] (reviewed in [28]). These Frzbs do not efficiently inhibit Wnt-3a activity [41•,48•], in spite of its 'Wnt-1-like' activity in separate assays [46,47]. This result suggests that Frzb proteins discriminate between Wnt proteins that seem functionally similar.

It is interesting to note that although Frzb-1 seems to bind to Wnt-5a it does not seem to interfere with signaling mediated by Wnt-5a [37•,48•]. The observation that association of the Frzb and Wnt proteins does not necessarily inhibit Wnt function was also demonstrated by examination of the activity of various Frzb deletion constructs [37•]. Although mutants of Frzb-1 with small deletions in the CRD bind Wnt-1 in co-immunoprecipitation assays, they do not seem to efficiently interfere with Wnt-1 signaling [37•].

Cerberus antagonism: is it all in your head?

Cerberus is a secreted factor expressed in the *Xenopus* gastrula organizer and is thought to have a role in head induction [49]—a process inhibited by ectopic Wnt-8 signaling in the gastrula dorsal mesoderm [50]. Cerberus has recently been shown to be a potent inhibitor of Wnt signaling in early *Xenopus* embryos, though the mechanism of inhibition is not understood [3•]. As the *Cerberus* expression domain overlaps that of *Frzb-1*, it seems that the gastrula organizer is a source of multiple inhibitors of Wnt signaling, which likely serve to sharpen the boundaries of cell fate (reviewed in [4]; see also Note added in proof).

The enemy within: Xwnt-5a?

The Frzbs proteins may be lurking impostors of Frizzled receptors lying in wait for stray Wnt proteins, but the embryonic responses to some Wnt proteins may also be blocked by members of their own family. It has become clear that the Wnt and Frizzled proteins can transduce at least two distinct types of signal [2,29•,30••,34••,46,47] (reviewed in [28,32]). In various assays, Wnt proteins can be assigned to the 'Wnt-1 class', which transduces signals via the pathway in Figure 1, or to the Wnt-5a class [47], which activates the phosphatidylinositol (PI) cycle via a G-protein coupled pathway [30••]. Members of the Wnt-5a class can inhibit embryonic responses to members of the Wnt-1 class, at least in early *Xenopus* embryos [2]. This inhibition is likely indirect and can be mimicked by stimulating the PI cycle and calcium signaling by a serotonin receptor [29•]. It is worth noting that although Wnt-5a and related Wnt proteins do not stabilize β-catenin (β-cat) in *Xenopus* embryos before the mid-blastula transition (MBT; the onset of zygotic transcription), Wnt-5a signaling after MBT leads to the stabilization of β-catenin [51•]. This shift in signaling identity is probably the result of the expression of a distinct member of the Frizzled family after MBT, perhaps Frizzled-5 [14•], which can bind Wnt-5a class ligands and transduce a Wnt-1-like signal [51•].

The nuts and bolts of β-catenin regulation

Modest increases in levels of β-catenin are sufficient to activate transcription of Wnt-responsive genes in embryos [24•,51•,52•]. Phosphorylation of β-catenin on conserved amino-terminal serine and threonine residues by GSK-3β [7] may be all that is required to target the protein for ubiquitination and proteasome degradation [9]; however, others have suggested that β-catenin/Armadillo may be a poor substrate for GSK-3β *in vitro* [8,53] and suggest an alternative mechanism involving the adenomatous polyposis coli protein (APC). APC has been implicated, at least in tumor cell lines, in the regulation of β-catenin levels [8], however, *Drosophila* embryos lacking zygotic APC regulate Armadillo appropriately [54] (reviewed in [55]). A new player, Axin, may also have a role in this part of the pathway, as described below.

As mutations at the *Fused* locus (as distinct from *Drosophila fused* mutants) were known to cause twinning of the axis in homozygous mouse embryos (reviewed in [56•]), investigators were interested in determining if Axin, the protein encoded at the locus, was involved in regulation of signaling pathways known to cause axis duplications in *Xenopus* embryos [56•]. Overexpression of Axin in cleavage-stage *Xenopus* embryos inhibited the ability of Wnt, Dishevelled, or a dominant-negative mutant of GSK-3β to promote formation of an ectopic dorsal axis. In contrast, Axin did not block axis induction in response to β-catenin or Siamois, a homeodomain-containing protein induced by Wnt signaling in the early *Xenopus* embryo [13,56•,57,58]. Other factors known to cause axis duplication in *Xenopus* such as Noggin, Activin and a dominant negative form of the BMP receptor were also unaffected by Axin. A mutant of Axin lacking a carboxy-terminal domain with identity to RGS proteins (AxinΔRGS, regulators of G-protein signaling, reviewed in [59]), behaves like a dominant negative and results in axial duplication when ectopically expressed. Co-expression of cadherin, which may inhibit Wnt signaling by sequestering β-catenin from the free cytoplasmic signaling pool, blocks axis induction by this mutant, suggesting that AxinΔRGS causes axis duplication by increasing the free signaling pool of β-catenin [56•]. The mechanism of Axin action is not known, and biochemical studies will certainly add to our understanding of its regulation of β-catenin.

LEF/TCF: architects of transcription

Although the nuclear translocation of β-catenin had been thought to correlate with its signaling function in the

Wnt pathway, it has only recently become clear how this activity is transduced. Two-hybrid screens revealed interactions between the LEF/TCF family of architectural transcription factors and β-catenin [19,20]. Complexes of LEF/TCF and β-catenin/Armadillo have been shown to bind to consensus LEF/TCF sites in promoters of several genes [23,24•,60] and in synthetic constructs [19,20]. Interactions are mediated by the conserved amino termini of LEF/TCF family members and arm repeats 3–8 of β-catenin/Armadillo [19,20,22•,23]. In *Drosophila*, *dTCF/pangolin* seems to be required in all *wingless*-dependent signaling events examined [22•,23,61••].

In spite of the sequence similarity that LEF and TCF family members retain in their amino termini and in their highly conserved HMG box DNA-binding domains, these proteins are not functionally equivalent. Overexpression of LEF-1 in *Xenopus* [20] and *Drosophila* [23] results in the activation of Wnt signaling pathways, whereas overexpression of *Xenopus* Tcf-3 [19] or *Drosophila* dTCF/Pangolin [22•] do not.

Association of β-catenin/armadillo with LEF/TCF activates transcription from endogenous and synthetic promoter fragments [19,22•,24•]. This activation could, in principle, be the result of a release from LEF/TCF-mediated transcriptional repression (reviewed in [62]). Indeed, it has been suggested that the LEF/TCF transcription factors are bound to promoters in the absence of β-catenin/Armadillo and repress basal transcription [23,24•]. Also, in an early polarizing event in *C. elegans*, Wnt signaling and LEF/TCF function seem to have strictly antagonistic roles [33,34••] (reviewed in [63]). This model is not sufficient, however, to explain all the details of β-catenin/Armadillo-dependent transcriptional activation in *Drosophila* and vertebrates. Studies in yeast [23] and mammalian cells [22•] have shown that chimeras of a carboxy-terminal region of β-catenin/Armadillo with heterologous DNA-binding domains are sufficient to activate transcription from synthetic minimal promoters. Taken together, these studies suggest that association of β-catenin/Armadillo with LEF/TCF both relieves transcriptional repression by LEF/TCF and activates transcription by the carboxy-terminal β-catenin/Armadillo domain. It is important to note that both promoters studied to date required other factors for maximal expression [23,24•].

Conclusions

Our understanding of the positive and negative regulation of Wnt signaling has broadened tremendously in the past 18 months. Wnt receptors (the Frizzled family), secreted antagonists (Frzb proteins and Cerberus), kinases (CKII, PKC), proteins of less clearly understood function (APC, Axin), and transcription factors (LEF/TCF) have been identified, and the biochemical relationships among these components are being elucidated. There are, no doubt, essential components of these pathways that have yet to be identified, and the relationship between the 'classical' signal transduction pathway (Figure 1) and divergent Wnt and Frizzled signaling pathways will continue to be clarified. Finally, systematic studies must be undertaken to determine which members of the Wnt and Frizzled families can interact in a productive manner and whether members of the other multi-gene families involved in the transduction of Wnt signals are generally interchangeable.

Note added in proof

An additional secreted antagonist of Wnt signaling has recently been cloned, and found to be expressed in the Spemann Organizer. Its mechanism of action remains unclear [64].

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